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22. MASAMUNE, H., M. SUZUKI AND Y. KONDOH. *J. Biochem. (Japan)*. 31: 343, 1940.
23. WOLFROM, M. L., D. I. WEISBLAT, J. V. KARABINOS, W. H. MCNEELY AND J. MCLEAN. *J. Am. Chem. Soc.* 65: 2077, 1943.
24. WOLFROM, M. L. AND W. H. MCNEELY. *J. Am. Chem. Soc.* 67: 748, 1945.
25. JORPES, J. E., H. BOSTRÖM AND V. MUTT. *J. Biol. Chem.* 183: 607, 1950.
26. MEYER, K. H. AND D. E. SCHWARTZ. *Helv. Chim. Acta* 33: 1651, 1950.
27. BURSON, S. L., JR. M. J. FAHRENBACH, L. H. FROMMELT, B. A. RICCARDI, R. A. BROWN, J. A. BROCKMAN, H. V. LEWRY AND E. L. R. STOKSTAD. *J. Am. Chem. Soc.* 78: 5874, 1956.
28. WOLFROM, M. L., T. M. SHEN AND C. G. SUMMERS. *J. Am. Chem. Soc.* 75: 1519, 1953.
29. KANTOR, T. G. AND M. SCHUBERT. *J. Am. Chem. Soc.* 79: 152, 1957.
30. WOLFROM, M. L., R. MONTGOMERY, J. V. KARABINOS AND P. RATHGEB. *J. Am. Chem. Soc.* 72: 5796, 1950.
31. WEISSMANN, B. AND K. MEYER. *J. Am. Chem. Soc.* 74: 4729, 1952.
32. MASAMUNE, H., N. HIYAMA AND T. KOBAYASHI. *Tohoku J. Exper. Med.* 44: 33, 1942.
33. DAVIDSON, E. A. AND K. MEYER. *J. Am. Chem. Soc.* 76: 5686, 1954.
34. FOSTER, A. B., D. HORTON AND M. STACEY. *J. Chem. Soc.* 1957, p. 81.
35. JEANLOZ, R. W. *Experientia* 6: 52, 1950.
36. FOSTER, A. B., E. F. MARTLEW AND M. STACEY. *Chemistry & Industry* 1953, p. 825.
37. MEYER, K. H. AND H. WEHRLI. *Helv. Chim. Acta* 20: 361, 1937, and ref. 2., p. 353.
38. STACEY, M. In: *Chemistry and Biology of Mucopolysaccharides*. Ciba Foundation Symposium, London: Churchill, 1958, p. 4.

PHYSICAL CHEMISTRY OF HYALURONIC ACID^{1,2}

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IN STUDYING the physicochemical characteristics of a polymer-type macromolecule, a constituent of solid tissues, the basic questions are: 1) What changes, if any, has the molecule undergone in the course of the preparation procedure? 2) How representative is the purified sample of the macromolecules of the tissue? Since hyaluronic acid is a building block of connective tissue, these points must be considered in studying its molecular parameters.

The two questions propounded cannot be answered at the outset of such a study, and therefore, certain assumptions must be made. One is that the purified polysaccharide, free of all proteins, is the molecular entity on which physicochemical studies should be carried out. Another assumption is that by removing all of the proteins one may break up a naturally-occurring molecular complex, the physicochemical characterization of which is of primary importance. The former stresses the purification process and is less concerned with possible

degradation in the course of preparation with the yield of the method. The latter emphasizes the importance of a mild preparation method and disregards the consideration of physicochemical studies should, preferably, be made only on molecular entities which have been chemically well characterized. Both lines of thought are well represented in the literature of the last ten years on the physical chemistry of hyaluronic acid.

Since hyaluronic acid can be prepared from proteins, with a fairly high degree of polymerization retained, this discussion deals with the physical chemistry of this polysaccharide. The purity of the hyaluronic preparations used in the studies reported in this paper was tested by hexosamine and nitrogen determinations. When the hexosamine-hexuronic acid ratio is close to one and the nitrogen content is not much higher than would be expected from the hexosamine content, it is assumed that the preparation is protein-free. Preparations with a protein content of up to 5% were often used in physicochemical studies. This protein content was calculated on the assumption that the hexosamine nitrogen represents pro-

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tain 16% nitrogen. Sulfur and galactosamine determinations were also used to check the presence of sulfated polysaccharides. Several physicochemical investigations were reported on preparations which contained up to 7% of unidentified sulfated polysaccharides. Many more have never been checked for the presence of this possible impurity.

The significance of the presence of proteins in an amount of less than 5%, from the viewpoint of molecular configuration, is unknown. There is no way to tell, at this time, what the nitrogen content in some preparations indicates. It may result from incomplete hydrolysis before hexosamine determination or from other errors in hexosamine analysis. It may originate from the presence of bacteria or from proteins completely unrelated to the polysaccharide. On the other hand, it may represent peptide bridges cross-linking the polysaccharide chains.

Our present knowledge about the physical chemistry of hyaluronic acid will be reviewed in a discussion of such molecular parameters as weight, dimensions, shape, volume and hydration. The charge effect on the structure of this polymer and its interactions will be mentioned briefly.

MOLECULAR WEIGHT

The molecular weight of hyaluronic acid is in the range of 50,000 to 8×10^6 , depending

on the source and on the methods of preparation and determination. The lowest polymer is that prepared from the bovine vitreous body, which gives a weight average molecular weight of less than 1×10^6 . Hyaluronic acid prepared from bovine synovial fluid, human rheumatoid arthritic synovial fluid, human umbilical cord, pig skin and rooster comb exhibits a weight average molecular weight of well over 1×10^6 .

Whether or not the hyaluronic acid prepared represents the average molecule of the tissue depends greatly on the yield of the method of preparation and on how well the total molecular population is represented in the sample. In order to evaluate the hyaluronic acid preparation from this aspect, one must know its concentration in the tissue. Such an evaluation can be made only on the bovine vitreous body because of lack of data on other tissues.

Molecular weight determinations on hyaluronic acid prepared from the bovine vitreous body have been reported by various authors, using the methods of osmotic pressure, sedimentation-diffusion and light scattering (table 1). The methods of preparation used to obtain pure hyaluronic acid from various tissues can be divided into two types: One is the removal of the proteins present in the tissue or in the tissue extract by means of proteolytic enzyme treatment, by selective precipitation or by extraction methods. The other is based on the fact that the electrophoretic mobility of hyaluronic acid is

TABLE 1. MOLECULAR WEIGHT OF HYALURONIC ACID PREPARED FROM BOVINE VITREOUS BODY

Methods of Preparation	Approx. Yield %	Molecular Weight $\times 10^{-5}$			Reference
		Osmotic Pressure (\bar{M}_n)*	Sedimentation, Diffusion (\bar{M}_w)†	Light Scattering (\bar{M}_w)†	
Removal of proteins by chloroform extraction and rosin and NaCl precipitation	20-30 50-60		245 87 (13)	500 340-500 (4, 8)	(10) (4, 8, 13)
Ethylene sulfonate precipitation	20-30		296 (10)	1270 (11)	(10, 11)
Enzymatic hydrolysis					
Pepsin-trypsin, trichloro-acetic acid precipitation	100		57		(10)
Papain, cetylpyridinium	100				
Pancreas extract, trichloro-acetic acid precipitation		270		300	(4, 8) (35)
Removal of hyaluronic acid by electrophoresis			178 220		(12) (10)
Electrodeposition				680	(10)

* = number average molecular weight.

† \bar{M}_w = weight average molecular weight.

greater than that of most proteins. Separation of hyaluronic acid from the vitreous body and from synovial fluid by electrophoresis was first reported by Blix (1). However, he did not use electrophoretically prepared hyaluronic acid for physicochemical studies. Varga and Gergely (2) prepared hyaluronic acid from the bovine vitreous body, and this preparation was used for extensive physicochemical studies. The advantage of the electrophoretic separation method is that, most likely, the distribution of the molecules is the same in the purified fraction as in the tissue extract. The disadvantage of this method is that hyaluronic acid cannot be separated from sulfated mucopolysaccharides, which have a greater electrophoretic mobility than hyaluronic acid itself.

A new separation method, called electrodeposition, has been described recently by Roseman and Watson (3). This method is also based on electrophoretic mobility, but uses electro-dialysis machines and collects the hyaluronic acid which accumulates as a paste on the dialysis membrane toward the anode. The advantages of this method over electrophoretic separation are that large volumes can be handled and the sulfated mucopolysaccharides can be separated from hyaluronic acid. In most tissue extracts this method alone does not produce protein-free hyaluronic acid. Treatment with Celite was recommended in those cases to remove the last 20-30% of proteins from the sample.

The highest yield (approximately 100%) is obtained when the proteins are removed by pepsin hydrolysis. However, if pepsin is used, incubation at 37° in 0.1 N HCl is required, and this treatment partially depolymerizes the polysaccharide, although polymers small enough to be dialyzable are not produced. Digestion with papain or with crude extracts made from pancreas and intestine does not result in a protein-free hyaluronic acid. However, in combination with phenol, cetylpyridinium or trichloro-acetic acid treatment, the proteolytic enzyme digestion methods will result in preparations with a protein content of less than 5% (4-6).

Other methods have been described for the preparation of hyaluronic acid free from proteins, using chloroform to extract the proteins (7), or using acid resin (8) or xylene sulfonate (9) to precipitate them. All of these methods, however, result in a yield of only 30-60%. Molecular weight determinations indicate that with these different methods of preparation one

either degrades the hyaluronic acid in various degrees or one obtains fractions having various molecular weights.

The lowest molecular weight sample of hyaluronic acid ($57-70 \times 10^3$) was prepared from the bovine vitreous body by the pepsin-try digestion method. This preparation was so polydisperse that the light-scattering measurements could not be used for molecular weight calculations (10). The acid resin precipitation method also gave a sample of low molecular weight (87×10^3), calculated from sedimentation and diffusion data. Using this method of purification, approximately 50% of the hyaluronic acid precipitates with the proteins during acidification and deionization (8). It is possible that a higher polymer fraction of hyaluronic acid is removed with the protein by Chloroform extraction and xylene sulfonate precipitation of the proteins result in a high molecular weight hyaluronic acid fraction (10, 11). The molecular weight of hyaluronic acid prepared by the electrophoresis or the electrodeposition method is within the values mentioned previously ($136-240 \times 10^3$) (10, 12).

It is interesting to note that the molecular weights determined by light-scattering measurements are always higher than those determined by sedimentation or diffusion studies, probably because the light-scattering method is more sensitive to the polydispersity of the sample (8, 10).

A comparison of the molecular weights of hyaluronic acid prepared from bovine synovial fluid, human rheumatoid arthritic synovial fluid, human umbilical cord and rooster comb suggests that there is no significant difference in the size of the molecules obtained by different preparation methods (table 2). Most of the variation in the molecular weight, calculated from sedimentation and diffusion studies, is within the error of the determination. The sample which showed a significantly lower molecular weight was that prepared from rooster comb (10). Some preparations of hyaluronic acid happened to be studied only by light scattering and showed a much greater variation (table 2). It is not yet possible to determine whether the data here summarized indicate that hyaluronic acid is present in various tissues in different degrees of polymerization or whether the differences were artificially introduced during preparation. The great discrepancy sometimes found between the molecular weight calculated from

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TABLE 2. MOLECULAR WEIGHT OF HYALURONIC ACID PREPARED FROM VARIOUS CONNECTIVE TISSUES

Source	Method of Preparation	Molecular Weight $\times 10^{-6}$		Reference
		Sedimentation, diffusion	Light scattering	
vine synovial fluid	Papain, cetylpyridinium		1.6	(8)
	Ultrafiltration, papain	2.0		(14)
	Electrodeposition	1.57	1.8	(10)
	Electrodeposition	1.86	3.2	(10)
human synovial fluid (rheumatoid arthritis)				
human umbilical cord	Pepsin-trypsin		8.0	(20)
	Papain, cetylpyridinium		5.8	(8)
	Chloroform		3.4	(16)
	Proteolytic enzymes, phenol, cetylpyridinium		3.0	(8)
rooster comb	Electrodeposition	1.44	2.7	(10)
	Papain, cetylpyridinium		1.3	(8)
	Electrodeposition	5.13	1.3-4.2	(10)

sedimentation-diffusion studies and that calculated from light scattering suggests that each preparation may have a different degree of polydispersity.

DIMENSIONS, SHAPE, VOLUME, HYDRATION

The conclusions drawn about the dimensions and shape of the hyaluronic acid molecule greatly depend on the method used for its study. When the molecule is exposed to a shearing force in the course of measurement, as in the measurement of double refraction of flow, a deformation and uncoiling will occur. This deformation and uncoiling is more pronounced in the high molecular weight hyaluronic acid than in the low polymer prepared from the vitreous body (7, 9-11).

The calculated dimensions of the molecule depend greatly on the shape assigned to it. The axial ratio (l/d) and the length calculated from data based on viscosity, sedimentation, diffusion, double refraction of flow and light-scattering studies on hyaluronic acid prepared by various methods from different sources, are tabulated in tables 3 and 4. The length of the hyaluronic acid molecule prepared from the vitreous body is between 1600 and 3400 Å, depending upon the method of preparation used. The lowest figures are observed in hyaluronic acid prepared by pepsin-trypsin digestion. This preparation also has the lowest molecular weight (57,000), and its length is that of an almost completely stretched molecular chain. Blix and Snellman in their viscosity and double refraction of flow studies derived a molecular length of 1000-

4800 Å, assuming that the molecule is a stretched polysaccharide chain (7). Both light scattering and double refraction of flow experiments, assuming a prolate ellipsoid of revolution or a randomly-kinked coil, gave shorter lengths and indicated that the molecular chain is not stretched (2, 4, 8, 10-13).

In the hyaluronic acid samples of higher molecular weight (table 4) the axial ratio calculated from sedimentation-diffusion studies is quite large (700-2600). This dissymmetry is based on the frictional ratio, assuming that the molecule is not hydrated. The dissymmetry calculated from viscosity is somewhat lower, but it also involves the assumption that there is no hydration. The ellipticity of a prolate ellipsoid of revolution, calculated from sedimentation and viscosity data by Blumberg and Ogston (14), was found to be 7.7. From light-scattering and viscosity measurements other authors (10) found an even lower dissymmetry in the high molecular weight hyaluronic acid preparations. All this indicates that the axial ratio of the large hydrated molecules is very low, but that of the lower polymers prepared from the vitreous body is considerably higher.

All light-scattering studies indicate that hyaluronic acid is a randomly-kinked coil with more or less polydispersity and stiffness (4, 8, 10, 11, 15, 16). The length of the molecule calculated from the radius of gyration, assuming the above model, varied between 2400 and 6400 Å in the different high molecular weight samples (table 4). This variation may be partially due to the polydispersity of the preparations. Laurent (4) tried to correlate the radius of

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Method of Preparation	[η]*	Molecular Model					Reference	
		Prolate ellipsoid of revolution		Random coil	Rigid rod†			
		Axial ratio (l/d) calculated from		Length in A calculated from				
		viscosity	frictional ratio	double refraction of flow	light scattering	double refraction of flow		
Chloroform extraction	200	70				1000	(7)	
	460	115				2600	(7)	
	880	265				4800	(7)	
	700	138	105		2500		(10)	
Acid resin and NaCl pre- cipitation	230	70	62		3400		(4, 8, 13)	
Xylene sulfonate precipita- tion	700	138	230	2500	2100		(10, 11)	
Pepsin-trypsin, trichloro- acetic acid preparation	230	70	64	1605			(10)	
Papain, cetylpyridinium	380	95			2100		(4, 8)	
Electrophoresis	690	137	145	2080			(2, 12)	
Electrodeposition	640	131	180	2080	2500		(10)	

* Limiting viscosity number, concentration in grams per cc.

† Stretched polysaccharide chain with estimated width (d) of 12 Å.

TABLE 4. MOLECULAR DIMENSIONS OF HYALURONIC ACID PREPARED FROM VARIOUS CONNECTIVE TISSUES

TABLE 4. MOLECULAR WEIGHTS

Source	Method of Preparation	[η]*	Molecular Model				Reference
			Prolate Ellipsoid of revolution		Random coil		
			Axial ratio (l/d) calculated from		Length in A calculated from		
			viscosity	frictional ratio	double refraction of flow	light scattering	
Bovine synovial fluid	Papain, cetylpyridinium	910	170			3900	(8)
	Ultrafiltration, papain	2200	$p = 7.7†$				(14)
	Electrodeposition	4830	391	700			(10)
Human synovial fluid (rheumatoid arthritis)	Electrodeposition	3230	322	760	10,300	2900	(10)
Human umbilical cord	Pepsin-trypsin					2100 (sphere)	(20)
Rooster comb	Papain, cetylpyridinium	1330	200			6400	(8)
	Chloroform extraction	3360	327			5400	(16)
	Proteolytic enzymes, phenol, cetylpyridinium	2020	245			5000	(8)
	Electrodeposition	2990	310	520	9,180	2450	(10)
	Papain, cetylpyridinium	950	175			3900	(8)
	Electrodeposition	3130	318	2600	18,100	3700	(10)

* Limiting viscosity number, concentration in grams per cc.

† p = ellipticity.

gyration and the length calculated from it with the molecular weight, assuming that the degree of polydispersity was about the same in the different preparations. The correlation indicated

a certain degree of stiffness, or, in other words, nonsolvent-draining, randomly-coiled molecule. Laurent also calculated the statistical unit of the randomly-kinked coil according to Kuhn.

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and Kuhn (17) and found it to be 300 Å, containing 60 monosaccharide units. This indicates considerable stiffness of the glucosidic linkages which can be explained by several mechanisms, such as solvent-solute interaction, interaction with proteins present in small amounts in the preparation, or cross-linkages.

The problem of the hydration of the hyaluronic acid molecule is closely connected with the problem of molecular volume. It is obvious, from many experiments carried out on pure hyaluronic acid (10) and on hyaluronic acid preparations containing proteins (18, 19), that the hydrodynamic volume or the hydrodynamically effective volume is many times greater than that calculated from the dry partial specific volume. The effective volume of a random coil can be calculated as an equivalent rotational ellipsoid of the prolate type, which comprises, then, the whole domain of the molecule including the randomly-coiled molecular chain and the water in between and surrounding it, as well as whatever additional proteins or other materials are present within the domain. This effective hydrodynamic volume was calculated by various methods from light-scattering, sedimentation and viscosity measurements (10). In the case of low polymer hyaluronic acid prepared from the vitreous body by electrodeposition, the hydrodynamic specific volume was approximately 6, but in the case of large polymers, such as hyaluronic acid prepared from synovial fluid and umbilical cord by the electrodeposition method, this value was between 1000 and 1860. Other authors (14, 18-20) found similarly high values for the hydrodynamic specific volumes

both in high molecular weight hyaluronic acid and in hyaluronic acid, containing 20-30% protein, prepared from synovial fluid by ultrafiltration.

This large volume of water in the domain of the hyaluronic acid coil is not bound to the polysaccharide by long-range forces, as suggested by Jacobson and Laurent on the basis of dielectric studies (21). Laurent showed recently (22) that the x-ray diffractogram of water does not change in the presence of 2% hyaluronic acid as one would expect if hydration-water in this amount was bound to the polysaccharide chain.

A new approach to the problem of the solvation and shape of the hyaluronic acid molecule was introduced by Laurent in studying the physical parameters of cetylpyridinium (CP) hyaluronate (umbilical cord) dissolved in methyl alcohol by the light-scattering method (23). These studies were later supplemented by the experiments of Varga, Pietruszkiewicz and Ryan (24) on CP-hyaluronate prepared from the vitreous body and investigated by sedimentation, diffusion and double refraction of flow methods. Table 5 summarizes their results. The molecular weight of CP-hyaluronate increases as expected. The radius of gyration and the length of CP-hyaluronate in methyl alcohol is approximately half that of Na-hyaluronate in water, indicating a coiling up of the chain. The decrease of the viscosity and the dissymmetry supports this finding. The length measured by double refraction of flow increases, suggesting that under the influence of the shearing force the molecule becomes readily uncoiled. All this points to the importance of the solvent-solute

TABLE 5. MOLECULAR PARAMETERS OF CETYLPYRIDINIUM HYALURONATE IN METHYL ALCOHOL

		Light Scattering		$[\eta]^*$	Sedimentation-Diffusion			Double Refraction of Flow
		$M \times 10^{-3}$	Radius of gyration A		$M \times 10^{-3}$	1/d	Length	Length
							$\frac{A}{f/f_0}$	A
Human umbilical cord†	Na-HA	3.0	2030	2020				
	CP-HA	5.4	1185	670				
	Na-HA	3.0	1800	1360				
	(regenerated)							
Ovine vitreous body‡	Na-HA				178	145	2100	2080
	CP-HA				289	30	900	2400

* Limiting viscosity number, concentration in grams/cc.

† Laurent (23).

‡ Varga, Pietruszkiewicz, and Ryan (24).

interaction and to the effect of charged groups on the molecular configuration of hyaluronic acid.

The molecular and atomic configuration of hyaluronic acid is not well known and both electron microscope and x-ray diffraction studies are inconclusive. Electron microscope studies (11, 25, 26) suggest a filamentous molecular chain. X-ray diffractograms (27) showed four peaks corresponding to 1.3-1.5 Å, 2.5 Å, 5 Å and 10 Å interatomic distances. Laurent suggests that the 1.3-1.5 Å peak corresponds to carbon-carbon and carbon-oxygen bonds, the 2.5 Å peak to the distance of the second neighboring atom, and the 5 and 10 Å peaks represent the distance between two glucosidic bonds or that between chains.

CHARGES

The hyaluronic acid molecule is negatively charged when the carboxyl groups on the glucuronic acid moiety are dissociated. Titration studies were made by Jeanloz and Forchielli (28), conductometric and potentiometric titrations by Pantlitschko (29) and titrations at different ionic strengths by Laurent (15). The intrinsic dissociation constant was found by Laurent (15) to be 3.21, which is close to 3.33 (the dissociation constant of glucuronic acid). From electrophoretic mobility data Varga (13) calculated the effective charge of hyaluronic acid at neutral pH. In hyaluronic acid prepared from the vitreous body, of the 216 anionic groups present/molecule only 14 are effective at 0.12 ionic strength, but at 0.02 ionic strength this figure becomes 173, i.e. 80% of the total. The decreased shielding effect of the small ions will influence the shape of the molecule, resulting in uncoiling and possible changes in the solvent-solute interaction which, since we are dealing with a random coil, means changes in the solvent draining through the domain of the molecule. Both the length measured by light scattering (8) and the length calculated from double refraction of flow (11) increase at low ionic strength. Changes in the sedimentation and diffusion properties of hyaluronic acid at low ionic strength can be explained on the same basis (12, 13).

The polyelectrolyte character of hyaluronic acid is clearly shown by its viscous behavior, the dependence of which on ionic strength has been studied by several authors (7, 8, 30, 31). It has also been shown (8) that the viscosity-

concentration relationship at various strengths follows the Fuoss equation (32) characteristic of polyelectrolytes.

MOLECULAR INTERACTIONS

The interaction among the hyaluronic molecules in aqueous solution can be observed with all of the physicochemical methods used for the characterization of this molecule. The concentration dependence of the viscosity (18, 33), of the sedimentation and diffusion coefficients (10, 12, 13) and of the electrophoretic mobility (13) all indicate intermolecular interaction. The concentration dependence of the extinction angle in double refraction of experiments (2) and the concentration dependence of the angular dissymmetry of light scattering at low ionic strength indicate aggregation of the molecules at higher concentration (16).

The shear dependence of the limiting viscosity number of various hyaluronic acid preparations was studied with both rotating cylinder (18, 33, 34) and capillary-type (30) viscometers. These studies indicate that the deformability of the internal interaction of the hyaluronic molecules is very small.

The solvent-solute interaction in various hyaluronic acid preparations has also been studied by correlating the molecular weight, the limiting viscosity number and the radius of gyration (10). These studies suggest that the solvent draining or nonsolvent-draining character of the hyaluronic acid molecule depends somewhat on the method used for preparation and possibly on the purity of the preparation as well.

REFERENCES

1. BLIX, G. *Acta physiol. scandinav.* 1: 29, 1957.
2. VARGA, L. AND J. GERGELY. *Biochim. et biophys. acta* 23: 1, 1957.
3. ROSEMAN, S. AND D. WATSON. Abstracts of the Ninth International Congress on Rheumatic Diseases, Toronto, June 1957, p. 29.
4. LAURENT, T. C. *Arkiv för Kemi* 11: 487, 1957.
5. GARDELL, S. *Arkiv för Kemi* 4: 449, 1952.
6. SCOTT, J. E. *Biochem. J.* 62: 31, 1956.
7. BLIX, G. AND O. SNELLMAN. *Arkiv för Mineral. Geol.* 19A: 1, 1945.
8. LAURENT, T. C. *J. Biol. Chem.* 216: 263, 1955.
9. ROWEN, J. W. AND R. BRUNISH. *UCRL-1040*, Oak Ridge: U.S. Atomic Energy Commission Technical Information Service, 1955.
10. BALAZS, E. A., L. VARGA AND J. GERGELY. Abstracts of the Ninth International Congress on Rheumatic Diseases, Toronto, June 1957, p. 30.
11. ROWEN, J. W., R. BRUNISH AND F. W. B. *Biochim. et biophys. acta* 19: 480, 1956.

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- VARGA, L., A. PIETRUSZKIEWICZ AND M. RYAN. *Biochim. et biophys. acta* In press.
- VARGA, L. *J. Biol. Chem.* 217: 651, 1955.
- BLUMBERG, B. S. AND A. G. OGSTON. *Biochem. J.* 66: 342, 1957.
- LAURENT, T. C. *Akademisk Avhandling*. Uppsala: Almqvist, 1957.
- LAURENT, T. C. AND J. GERGELY. *J. Biol. Chem.* 212: 325, 1955.
- KUHN, W. AND H. KUHN. *Helvet. chim. acta* 26: 1394, 1943.
- OGSTON, A. G. AND J. E. STANIER. *Biochem. J.* 40: 585, 1951.
- FESSLER, J. H., A. G. OGSTON AND J. E. STANIER. *Biochem. J.* 58: 656, 1954.
- BLUMBERG, B. AND G. OSTER. *Science* 120: 432, 1954.
- JACOBSON, B. AND T. C. LAURENT. *J. Colloid Sc.* 9: 36, 1954.
- LAURENT, T. C. *Arkiv för Kemi* 11: 503, 1957.
- LAURENT, T. C. *Arkiv för Kemi* 11: 497, 1957.
24. VARGA, L., A. PIETRUSZKIEWICZ AND M. RYAN. *J. Polymer Sci.* In press.
25. JENSEN, C. E. AND F. CARLSEN. *Acta Chem. Scandinav.* 8: 1357, 1954.
26. GROSS, J. *J. Biol. Chem.* 172: 511, 1948.
27. LAURENT, T. C. *Arkiv för Kemi* 11: 513, 1957.
28. JEANLOZ, R. W. AND E. FORCHIELLI. *J. Biol. Chem.* 186: 495, 1950.
29. PANTLITSCHKO, M. *Monatsh. Chem.* 83: 1125, 1952.
30. SUNDBLAD, L. *Acta Soc. med. upsal.* LVIII: 113, 1953.
31. BALAZS, E. A. AND T. C. LAURENT. *J. Polymer Sci.* 6: 665, 1951.
32. FUOSS, R. M. AND A. S. FUOSS. *Ann. Rev. Physical Chemistry* 4: 66, 1953.
33. OGSTON, A. G. AND J. E. STANIER. *Biochem. J.* 48: 364, 1950.
34. OGSTON, A. G. AND J. E. STANIER. *Biochem. J.* 52: 149, 1952.
35. CHRISTIANSEN, J. A. AND C. E. JENSEN. *Acta Chem. Scandinav.* 9: 1405, 1955.

METABOLISM OF HYALURONIC ACID AND CHONDROITINSULFURIC ACIDS¹

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AN INCREASING NUMBER of investigations have been concerned recently with the metabolism of acid mucopolysaccharides. Most studies have employed S^{35} to investigate the *in vivo* metabolism of sulfated polysaccharides. A more limited number of studies have been concerned with *in vivo* turnover rates employing carboxyl labeled acetate and labeled glucose as precursors. While such studies yield considerable information regarding mucopolysaccharide metabolism, the information regarding pathways of biosynthesis is obtained by these techniques.

Although connective tissues are widely distributed throughout the mammal, it is difficult to obtain tissues with a sufficient density of connective tissue cells to be appropriate for studies of biosynthetic pathways. Since the capsular polysaccharide of group A streptococci appears

to be identical with mammalian HA² (1), this microorganism affords a convenient tool for the study of biosynthesis of HA.

During the past 7 years studies in this laboratory have been concerned with the elucidation of this biosynthetic pathway. It is the purpose of this communication to summarize these studies together with certain evidence concerning the metabolism of acid mucopolysaccharides in mammals. The early studies, conducted in collaboration with Dr. Saul Roseman, were con-

¹ The following abbreviations are used in this paper: hyaluronic acid, HA; chondroitinsulfuric acid, CSA; uridine diphosphoglucose, UDPG; uridine diphosphoglucuronic acid, UDPGA; uridine diphospho-N-acetylglucosamine, UDPAG; uridine diphospho-N-acetylgalactosamine, UDPAGA; acetylglucosamine-6-phosphate, AG-6-P; uridine triphosphate, UTP; acetylglucosamine-1-phosphate, AG-1-P; adenosine triphosphate, ATP; hyaluronic acid synthesizing system, HASS; cytidine triphosphate, CTP; guanosine triphosphate, GTP; diphosphopyridine nucleotide, DPN.

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